Video Article

Measuring Hypopharyngeal Gland Acinus Size in Honey Bee (*Apis mellifera*) Workers

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Abstract

The nurse hypopharyngeal glands produce the protein fraction of the worker and royal jelly that is fed to developing larvae and queens. These paired glands that are located in the head of the bee are highly sensitive to the quantity and quality of pollen and pollen substitutes that the nurse bee consumes. The glands get smaller when nurses are fed deficient diets and are large when they are fed complete diets. Because nurse hypopharyngeal gland size is a robust indicator of nurse nutrition, it is essential that those studying honey bee nutrition know how to measure these glands. Here, we provide detailed methods for dissecting, staining, imaging, and measuring nurse bee hypopharyngeal glands. We present comparisons of unstained and stained tissue and data that were used to study the impact of pollen on gland size. This method has been used to test how diet impacts hypopharyngeal gland size but has further use for understanding the role of these glands in hive health.

Video Link

The video component of this article can be found at https://www.jove.com/video/58261/

Introduction

Honey bees are essential for agriculture because they pollinate a variety of crops that are consumed by humans and animals. Much attention has been paid to the decline of honey bee populations as colony losses hover around 30-40% each year in the United States[1] and 10–15% in Europe[2,3]. Multiple factors, including reduced access to high quality forage, likely act together to negatively impact honey bee health. Monoculture, drought, unsustainable beekeeping practices, and other factors decrease the diversity and amount of natural pollens available to colonies[4,5]. Because honey bees derive almost all of their dietary proteins and lipids from pollen, reduced access to pollen can severely limit individual and colony health.

The hypopharyngeal glands are secretory structures located in the bee's head between the eyes and the brain[6]. Under normal circumstances, the developmental and functional trajectory of the glands mirror that of the bee they are located in. At approximately 5–10 days of age, the bee performs nursing behaviors in the hive. At this same time, the hypopharyngeal glands reach their peak size and secretory capacity, producing the major protein fraction of the brood food or jelly fed to developing larvae and other adults, such as the queen. At this peak size the glands resemble a bunch of grapes where each grape is a discrete lobe structure known as the acinus (plural: acini). As the worker bee ages and takes on different tasks in the hive, the hypopharyngeal glands shrink and take on different functions, like breaking down sugars in nectar[7,8]. The hypopharyngeal glands are therefore correlated with the age of the bee and their age-associated task.

Nurse hypopharyngeal gland size is sensitive to the quantity and quality of protein in their diet[9,10,11]. When nurse bees are well-nourished, their glands are large. Whereas, the glands are small when the bee is deprived of pollen, particularly in the first week of adult development. In order to determine the nutritional status of a nurse bee, researchers typically measure the hypopharyngeal glands, either by directly measuring gland acinus size[11,12,13,14] or protein content[15,16] or by measuring the protein content[11] or fresh weight[17] of the entire head where they are located. Each method has its own pros and cons. We prefer the resolution obtained from measuring the gland acini, though this method can be challenging in two major ways. The first challenge is to properly identify and dissect the gland. The second is obtaining an accurate measure of each acinus. Under a dissecting light microscope, the glands appear clear or milky white and the borders of the acini can be difficult to define. Having tools to better define the edge of the acini, and to increase the likelihood of obtaining accurate gland measurements is beneficial to anyone studying honey bee nutrition.

Here, we show interested researchers how to dissect, stain, image, and measure hypopharyngeal glands so that accurate measurements of acinus size can be achieved. The method we describe offers researchers an easy, accurate, and replicable method for achieving multiple gland measurements in a relatively short period of time once the experimenter is sufficiently practiced. One could confidently measure the glands of almost 10 individuals in just over an hour. We offer details on both the method and materials needed to obtain these measurements. The most important aspects of the methods outlined below are the proper dissection and staining of the glands. Although we capture the magnified images and measure the acini with commercial software, the methods we present can easily be adapted to other platforms[18].

Protocol

1. Dissecting and Staining the Hypopharyngeal Glands from Nurse Workers

1. Make a wax dissection plate by melting cool setting wax into a small (60 mm x 15 mm) or large (100 mm x 15 mm) glass Petri dish. Cool the wax completely before using the plate for dissections.
2. For each bee to be processed, prepare 20 µL of a 1:20 working Giemsa solution (1:20 v/v of prepared Giemsa stain in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4)).
CAUTION: Giemsa stain contains methanol and is flammable. It is toxic if swallowed, inhaled, or if it comes in contact with skin. It should be disposed of according to the local institution's requirements.
NOTE: Always make a fresh working solution just for the current batch of samples because Giemsa stain quickly degrades once it is diluted. Discard the diluted stain if a precipitate develops.
3. Pipet 20 µL Giemsa stain into the wells of the microscope slides and 50–100 µL saline onto the slide adjacent to the well.
4. Detach the head from a bee, using the 10 mm micro-spring scissors, and embed the head front side up into the wax dissection plate using forceps and a wax-carving pen. Pin the head down into the wax plate for additional stability by inserting pins in the eyes and one in the mouth.
5. Using a sharp breakable razor blade fixed into a pin vise, make a small (~2–3 mm) incision between the eyes and mandibles on each side of the face plate. Gently run the micro scissors under the face plate and cut the antennal nerve that runs between the antennae and the brain.
6. Using fine forceps, grab the face plate by the mouth, flip it up, and pin it to the wax plate with fine point forceps and a pin. If needed, remove the face plate completely.
7. Pipet 20 µL PBS onto the cut open section of the head.
NOTE: The glands may float up at this point or one must search for them. The glands look like a string of pearls and are located on top of the brain if intact.
8. Use super fine point forceps to gently remove one of the hypopharyngeal glands (the gland may break, requiring it to be removed in segments), and immediately transfer the gland to the Giemsa stain on the microscopy slide.
9. Allow the gland to incubate in the Giemsa solution for 5 min, and then use forceps to transfer the gland to the pool of saline on the same slide. If needed, cut the gland into smaller pieces with the micro scissors.
NOTE: This helps to make the glands lie in a flatter plane, thus making it easier to obtain clear images of the gland acini.

2. Measuring the hypopharyngeal Gland Acini

1. Turn on the microscope and open the measurement program. Turn on the light source for the microscope if it is not already on. Set the microscope magnification to 10X.
2. Find the glands and focus the magnified image in the eyepiece, without the computer. Increase the magnification to 60–80X and focus the image in the eyepiece.
3. Under the '*Acquire*' tab, adjust and focus the glands on the live image on the computer.
4. Type image name/sample description into the 'Image Name' box and click 'Acquire Image' to take an image of the glands for further measurements.
5. Select the 'Analysis' tab. Select the 'Area tool' (highlighted in **Supplemental Figure 1A**) and unselect '*Value*' under '*Display Labels*', which will also unselect '*Unit*'. Set all other settings to default (**Supplemental Figure 1A**).
Note: The software automatically calibrates the area measurements according to the level of magnification so that the area obtained from a lower magnification is the same as that obtained from a higher magnification.
6. Measure each acinus by continuously clicking or continuously holding down the left mouse button while tracing the acinus perimeter as carefully as possible. Measure at least 10 acini per bee.
NOTE: More may be needed depending on the experiment (see the **Discussion**). It may require multiple images/sections of the gland to find enough clear, properly oriented acini for measurement.
7. Once all of the acini from a single image have been measured, select '*Create Report*'. Ensure that options are selected as shown in **Supplemental Figure 1B** and click '*Export*'.
8. Save the report as desired. If a file has already been created for the set of samples and additional measurements are being added, ensure that the report file is not open so that the new data are added to the existing file.
9. When finished, turn off the microscope camera and light source. Wipe the liquid and glands off the microscopy slides and safely dispose of any used material. Rinse the slides with distilled water and wipe clean with ethanol (70% v/v in water) for reuse.

Representative Results

Hypopharyngeal glands were dissected from nurse workers and visualized with and without stain at 60–80X magnification (**Figure 1**). In unstained tissue, it is difficult to find proper contrast to fully focus and define the edges of the acini. In the stained tissue, the edges of the acini are sharp because of the improved contrast between the stained tissue and the white background.

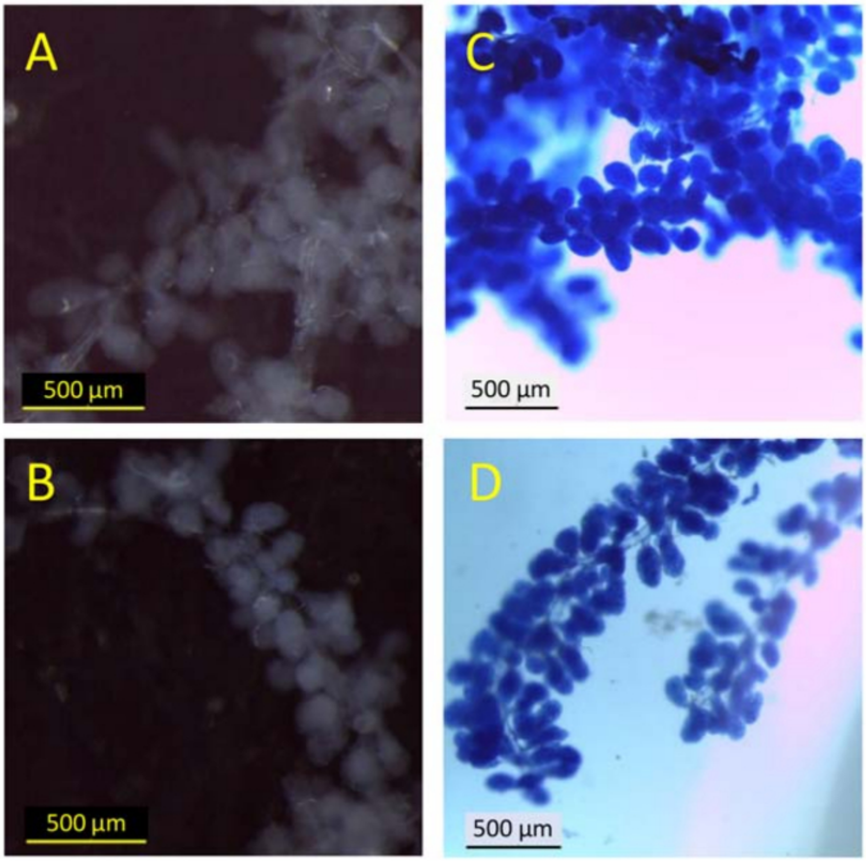


Figure 1: Unstained (A, B) or stained (C, D) hypopharyngeal glands from nurse-aged workers. Note that while the glands are visible in both cases, the stained glands are more well-defined and therefore easier to measure. Also note that the somewhat curled and coiled morphology of the whole gland results in different focal planes. The gland can be sectioned to prevent this from occurring. Scale bars = 500 µm. Please click here to view a larger version of this figure.

Honey bee workers were collected at ≤18 h after emergence and were assigned to two different dietary regimes: a diet of spring pollen naturally present in Tucson, Arizona, USA mixed with honey or just honey with no pollen. In order to restrict the bees to these diets while in the hive, push-in wire cages were used — as described in earlier studies[13,14] — confining the bees at a density of approximately one bee per square centimeter. Each dietary treatment was repeated in three hives (N = 3). Bees exposed to either dietary regime were collected at 5 d and 8 d of age for analysis of their hypopharyngeal glands. Using the dissection, staining, and measuring methods outlined above, the hypopharyngeal gland acini of these bees were measured and compared (**Figure 2**). In each of the three hives, three bees were measured for each age x diet treatment combination. Ten acini were measured for each bee. The acini measurements were averaged for each bee to obtain an average acinus size per bee. These values were then averaged to obtain a value for the age x treatment combination for that hive. ANOVA analysis on the acinus measurements showed that diet ($F_{1,8} = 2.65$, $p = 0.001$), age ($F_{1,8} = 10.03$, $p = 0.013$), and the interaction between diet x age ($F_{1,8} = 0.02$, $p = 0.020$) had significant effects. We observed that the hypopharyngeal glands grew over time in the bees that were fed pollen, as determined by a Tukey's HSD. This pattern has been demonstrated previously[9,11,19]. The size of the gland acini did not differ between the 5 d and 8 days old bees when the bees were deprived of pollen.

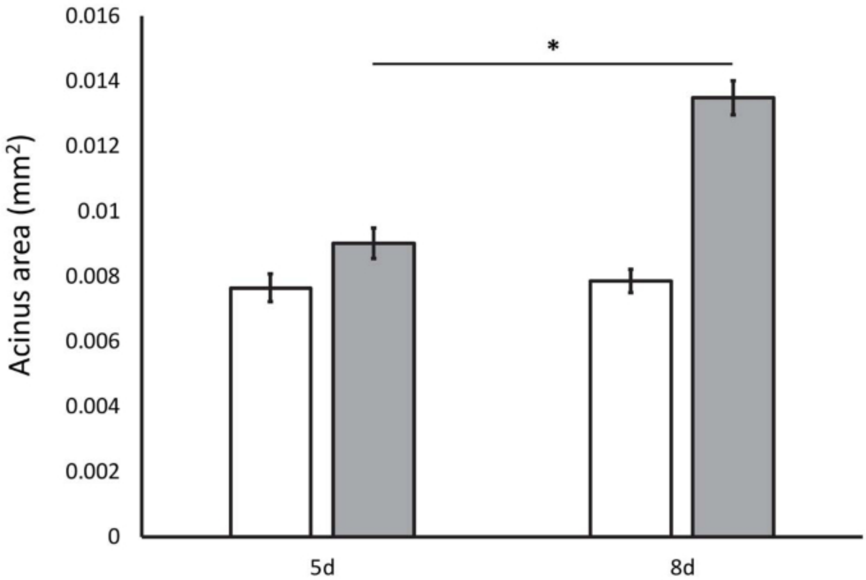


Figure 2: Hypopharyngeal gland sizes of well-fed nurses and those deprived of pollen. Workers were fed a diet of pollen and honey (gray bars) or a diet of honey alone (white bars) for 5 or 8 d. During this time, bees were inside of the hive and caged over honey or honey and pollen. Their hypopharyngeal gland acini were measured as described above. Error bars represent standard error for the mean acinus size across the three colonies (N = 3) tested. Three bees were measured from each colony for each treatment age x diet treatment combination. Bars connected by an asterisk are significantly different from each other according to a Tukey's HSD (α = 0.05). Please click here to view a larger version of this figure.

Bees can also be maintained in cages separate from the hive and fed defined diets. Honey bee workers were collected at ≤ 18 h after emergence and were assigned to acrylic glass cages (100 bees per cage) with one of four dietary regimes: a diet containing no pollen or a diet containing one of three bee-collected pollens: "almond" pollen from an almond orchard monoculture, "desert" pollen from the Sonoran Desert containing a mix of desert plants, or "SE" pollen from colonies located in the southeastern United States, as described in Corby-Harris *et al.*[12]. Sucrose (50% w/v), water, and pollen (where appropriate) were provided *ad libitum*. Five cages were constructed for each of the four dietary treatments, resulting in a total of 20 cages. At 8 d of age, the hypopharyngeal glands of ten bees were collected and measured. Ten acini were measured for each individual. The average acinus size was calculated for each bee and these values were used to obtain an average acinus size for each cage. We observed that hypopharyngeal gland size is sensitive to both the presence of pollen in the diet (pollen versus no pollen: $t_1 = 5.64$, $p < 0.0001$) and the type of pollen provided (almond *vs.* desert *vs.* SE pollen: $F_{2,148} = 8.06$, $p = 0.0005$; **Figure 3**). Bees fed desert or almond pollen had equivalent gland sizes. Bees fed SE pollen had glands that were smaller than bees fed almond or desert pollen.

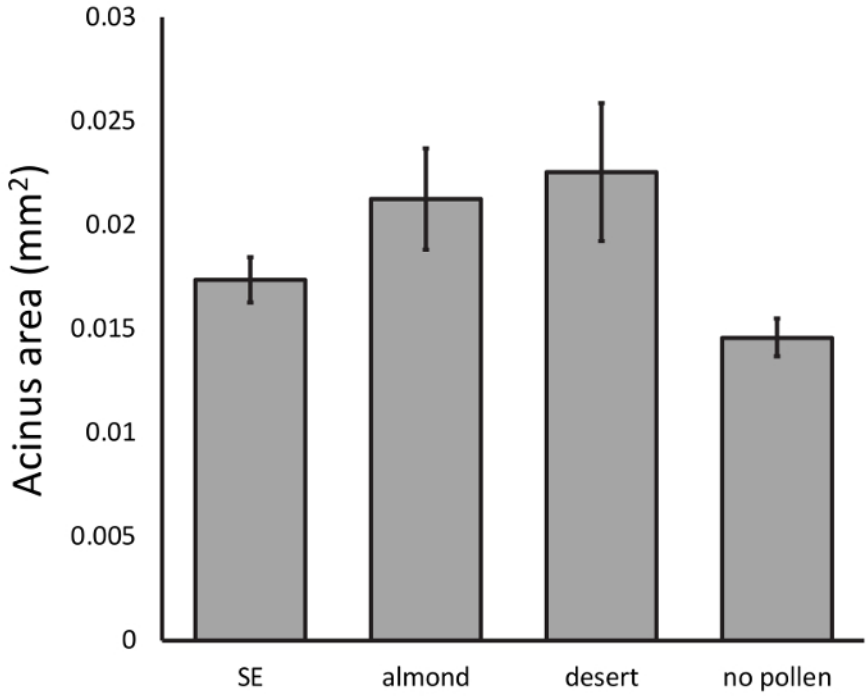
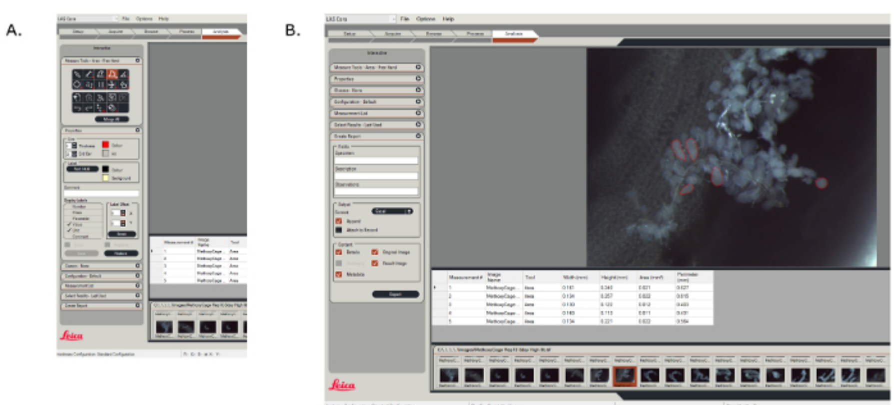


Figure 3: Hypopharyngeal gland sizes of nurse-aged workers fed three different types of pollen or deprived of pollen. Nurse bees were placed in cages after emergence and were fed a diet consisting of sucrose alone (no pollen) or sucrose and one of three pollens (almond, desert, or SE pollen) until 8 d of age. Error bars represent standard error for the mean acinus size in bees sampled from five cages (N = 5). Ten bees were sampled and measured in each cage to obtain an average acinus size for the cage and to calculate the variation among cages. Please click here to view a larger version of this figure.



Supplementary File 1: Screen shots of measurement software while measuring the gland acini (A) and creating the report (B). Please click here to view a larger version of this figure.

Discussion

Hypopharyngeal gland size is sensitive to the amount of protein and pollen in the diet and is a critical marker of nourishment in young adult bees. Here, we demonstrate an inexpensive and reproducible way to dissect and measure this tissue. These tissues can be difficult to dissect, but with practice, one can obtain increasingly cleaner dissections with the tissue relatively intact. The main advantage of the method presented here is that the tissue is stained, which enables the researcher to clearly visualize the borders of each gland acinus. Without a stain, the borders of these acini are difficult to visualize and focus under the microscope which lessens the researcher's ability to obtain an accurate acinus measurement. Despite the ease of staining and obtaining a clear image for the acini, several critical points should be considered in order to obtain accurate measurements; these are discussed below.

Large glands come from well-fed nurse bees that are approximately 7–10 days of age[9,20]. It is always easier, especially when learning to dissect these tissues, to first practice dissecting large glands before moving onto smaller glands as they can be small, fragile, and difficult to dissect. Fresh tissue also yields the best dissections. If one must freeze the bee for a period of time prior to the dissection, know that the longer that a bee is frozen, the more fragile the tissue becomes. This can make dissections problematic. With practice and sharp forceps, the researcher will eventually overcome these issues. We have not noticed a difference in staining between frozen and fresh tissue.

Fresh stain is necessary in order to obtain proper staining of the glands. Older stain can clump, leaving the stain unable to properly permeate the tissues. It is important that fresh stain be prepared in small batches, approximately every hour. This will ensure proper contrast between the tissue and the background under the microscope, which leads to sharp images with defined acinus edges. It is also helpful to work with a range of stain dilutions if the stain does not permeate the tissue properly. We found that a 1:20 fresh working stock of stain works best, but other dilutions can also work if one desires a darker or lighter stain.

We used a camera attached to the dissecting microscope and commercially available software to measure and record the acini data. The camera and software are somewhat costly and may therefore not be available to all labs. Although it is necessary to have a camera attached to the dissecting microscope in order to obtain the image and measure the acini there are several lower cost options, including free software[18], that can be used.

Here, we show the basic steps for staining and measuring the hypopharyngeal gland acini, but stress that it up to the researcher to decide how many acini to measure, whether to measure acini on one or both glands, and whether to measure multiple areas of each gland. For example, in order to detect more subtle differences among experimental treatments, one might need to measure more than 10 acini per gland and more than 10 bees per treatment. We have not noticed any acini size differences based upon their locations on the gland. We have also not noticed any differences in sizes of acini that are located on the left or right glands. If the researcher suspects any size differences, multiple areas of the hypopharyngeal gland and perhaps both glands should be measured.

Researchers should be able to successfully obtain accurate measurements of hypopharyngeal gland size using the protocol described here for dissecting and staining gland tissue and measuring acini. With practice, these measurements can be obtained rather quickly, allowing the researcher to process multiple samples in one sitting. We expect that more researchers will employ the methods outlined here as they seek to further understand the factors that influence hypopharyngeal gland size, and how these glands relate to colony health and individual behavior.

Disclosures

The authors have nothing to disclose.

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